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(54) Title: HEREGULIN–GAMMA

(57) Abstract

The present invention regards heregulin variants (HRG–gamma) with a truncated EGF domain and cDNA sequences encoding such variants. The present invention also regards the pharmaceutical application of such heregulins and diagnostic and therapeutic agents derived from such heregulins or their nucleotide sequences, respectively.

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Heregulin-Gamma

Technical Field

The present invention relates to a novel protein of the heregulin family, particularly to a heregulin which is truncated in the epidermal growth factor (EGF)-like domain.

Background Art

Heregulins (HRG) belong to a group of ErbB receptor family ligands which play an important role in regulating cell proliferation and differentiation of various normal and neoplastic tissues. The expression of particular heregulins as well as their receptors is tissue specific (1,2,3). HRG were discovered in different species and were isolated in part by their biological ability to activate the ErbB-2 cell surface tyrosine kinase receptor (4,5). They arise from alternative RNA splicing of two genes (6,7,8). All HRG contain an immunoglobulin (Ig)-like and an epidermal growth factor (EGF)-like domain, the latter existing in two variations denominated α and ß. To mediate ErbB receptor activation, the EGF-like domain is required (9), specified by small stretches of amino acids (10). HRG are glycosylated (30-40 %) heparin-binding molecules (6,11,12) produced as precursors containing a transmembrane domain with a cytoplasmatic tail of variable length. Their secretion occurs by insertion of the precursors into the plasma membrane and the release of the respective mature factor by specific proteolysis. Lack of the transmembrane domain impairs secretion (9).

Today, ErbB-2 inactivation is a novel strategy to treat advanced breast cancer, since ErbB-2 overexpression is correlated with poor prognosis (13) and loss of antiestrogen treatment efficacy in human breast cancer

(HBC) (14). In addition, HRG have been implicated in the progression of the disease (15).

Disclosure of the Invention

One object of the present invention was to isolate novel heregulin variants as a basis for an improved treatment of e.g. human breast cancer.

A further object of the present invention was to determine the nucleic acid structure of such heregulin variants.

Another object of the present invention was to provide a diagnostic agent for cancer diagnosis.

Still another object of the present invention was to provide methods for the production of therapeutic agents and such therapeutic agents themselves, particularly for breast cancer therapy.

Yet another object of the present invention was to provide a method for the regulation of cell activity, of cell activation processes and/or the stimulation/inhibition of cell proliferation, particularly of cells lacking an ErbB receptor.

By screening a cDNA library obtained from a HBC cell line a 1.6 kb clone of a novel heregulin splicing variant was isolated.

Said splicing variant is characterized by the coding sequence of amino acids 1 to 211 of heregulin terminated by a stop codon.

It was most surprisingly found that the coding sequence is flanked by a 568 bp 3'-untranslated region which has no correspondence in the cDNA sequences of heregulin-α as well as heregulin-ß.

It was also found that the 3'-untranslated region at its 5'-end (nucleotides 637 to 1207), i.e. on a length of 570 nucleotides is very similar or identical with the intron situated 3' of exon 6 which is hereinafter referenced as intron 6.

A sequence of a specific clone isolated is shown in Table I and the sequence listing.

In Table I the coding sequence (nucleotides 1 to 636 are marked in majuscules.

If nothing else is noted, the numbering of the sequences referred to herein furtheron starts with the coding sequence.

Table I

DNA sequence 1651 b.p. acggcacgagga ... aaaaaaaaaaaa linear

```
  -100    |   -90    |   -80    |   -70    |   -60    |   -50    |   -40    |   -30    |   -20    |   -10    |
                                                                                  acggcac gaggagccgg cgaggagttc cccgaaactt gttggaactc -401
-447
-400 cgggctcgcg cggaggccag gagctgagcg gcggcggctg ccggacgatg  ggagcgtgag caggacggtg ataacctctc cccgatcggg ttgcgagggc -301
-300 gccgggcaga ggccaggacg cgagccgcca gcggcgggac ccatcgacga  cttcccgggg cgacaggagc agccccgaga gccagggcga gcgcccgttc -201
-200 caggtggccg gaccgcccgc cgcgtccgcg ccgcgctccc tgcaggcaac  gggagacgcc cccgcgcagc gcgagcgcct cagcgcggcc gctcgctctc -101
-100 cccatcgagg gacaaacttt tcccaaaccc gatccgagcc cttggaccaa  actcgcctgc gccgagagcc gtccgcgtag agcgctccgt ctccggcgag -1
   1 ATGTCCGAGC GCAAAGAAGG CAGAGGCAAA GGGAAGGGCA AGAAGAAGGA  GCGAGGCTCC GGCAAGAAGC CGGAGTCCGC GGCGGGCAGC CAGAGCCCAG 100
 101 CCTTGCCTCC CCAATTGAAA GAGATGAAAA GCCAGGAATC GGCTGCAGGT  TCCAAACTAG TCCTTCGGTG TGAAACCAGT TCTGAATACT CCTCTCTCAG 200
 201 ATTCAAGTGG TTCAAGAATG GGAATGAATT GAATCGAAAA AACAAACCAC  AAAATATCAA GATACAAAAA AAGCCAGGGA AGTCAGAACT TCGCATTAAC 300
 301 AAAGCATCAC TGGCTGATTC TGGAGAGTAT ATGTGCAAAG TGATCAGCAA  ATTAGGAAAT GACAGTGCCT CTGCCAATAT CACCATCGTG GAATCAAACG 400
 401 AGATCATCAC TGGTATGCCA GCCTCAACTG AAGGAGCATA TGTGTCTTCA  GAGTCTCCCA TTAGAATATC AGTATCCACA GAAGGAGCAA ATACTTCTTC 500
 501 ATCTACATCT ACATCCACCA CTGGGACAAG CCATCTTGTA AAATGTGCGG  AGAAGGAGAA AACTTTCTGT GTGAATGGAG GGGAGTGCTT CATGGTGAAA 600
 601 GACCTTTCAA ACCCCTCGAG ATACTTGTGC AAGTAAgaaa agaaatcctg  tgtgtcgctt atgtctataa ctccttgttt cagatgattc tatgtctcat 700
 701 gattgattgt tgctttttt ccaattttgt tgcatcatgt tgaataatgc  tgttttatat gtagagtctt ttaaaacatt cacaccattc gtcatcactc 800
 801 ctctgtcata tgcagttttg ttttttgctc ttttcaatgt gtgtgaggtg  ttttttgttt ttgtttttgt ttttttgcca tgttatttat agtgttgctt 900
 901 tccttgtgct ttccttgtgg ttttcttggt tggttattca gaaaagatgt  gcagatatca cagaggccta tagccttttg gtatctactt ctacatccaa 1000
1001 tgtatgaatt aagctgtaag ataatgttgc tttcttatcc cagtgatcac  ctgccaaatg aataagacaa caaagagaag cagaagggca agaagattat 1100
1101 ttactgacat atatctatta cacttgggat tgtgcttact gttgcataac  tatttttaa acggagttta gttttatatt gctagtaaaa aaaaaaaaaa 1200
1201 aaaa                                                                                                        1204
        |   10    |   20    |   30    |   40    |   50    |   60    |   70    |   80    |   90    |   100
```

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
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(B) STREET: Auf dem Ruecken 1
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(D) STATE: Basel
(E) COUNTRY: Switzerland
(F) POSTAL CODE (ZIP): 4125

(ii) TITLE OF INVENTION: Heregulin-Gamma

(iii) NUMBER OF SEQUENCES: 1

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1651 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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ACGGCACGAG GAGCCGGCGA GGAGTTCCCC GAAACTTGTT GGAACTCCGG GCTCGCGCGG      60
AGGCCAGGAG CTGAGCGGCG GCGGCTGCCG GACGATGGGA GCGTGAGCAG GACGGTGATA     120
ACCTCTCCCC GATCGGGTTG CGAGGGCGCC GGGCAGAGGC CAGGACGCGA GCCGCCAGCG     180
GCGGGACCCA TCGACGACTT CCCGGGGCGA CAGGAGCAGC CCCGAGAGCC AGGGCGAGCG     240
CCCGTTCCAG GTGGCCGGAC CGCCCGCCGC GTCCGCGCCG CGCTCCCTGC AGGCAACGGG     300
AGACGCCCCC GCGCAGCGCG AGCGCCTCAG CGCGGCCGCT CGCTCTCCCC ATCGAGGGAC     360
AAACTTTTCC CAAACCCGAT CCGAGCCCTT GGACCAAACT CGCCTGCGCC GAGAGCCGTC     420
CGCGTAGAGC GCTCCGTCTC CGGCGAGATG TCCGAGCGCA AAGAAGGCAG AGGCAAAGGG     480
AAGGGCAAGA AGAAGGAGCG AGGCTCCGGC AAGAAGCCGG AGTCCGCGGC GGGCAGCCAG     540
AGCCCAGCCT TGCCTCCCCA ATTGAAAGAG ATGAAAAGCC AGGAATCGGC TGCAGGTTCC     600
AAACTAGTCC TTCGGTGTGA AACCAGTTCT GAATACTCCT CTCTCAGATT CAAGTGGTTC     660
AAGAATGGGA ATGAATTGAA TCGAAAAAAC AAACCACAAA ATATCAAGAT ACAAAAAAAG     720
CCAGGGAAGT CAGAACTTCG CATTAACAAA GCATCACTGG CTGATTCTGG AGAGTATATG     780
TGCAAAGTGA TCAGCAAATT AGGAAATGAC AGTGCCTCTG CCAATATCAC CATCGTGGAA     840
TCAAACGAGA TCATCACTGG TATGCCAGCC TCAACTGAAG GAGCATATGT GTCTTCAGAG     900
TCTCCCATTA GAATATCAGT ATCCACAGAA GGAGCAAATA CTTCTTCATC TACATCTACA     960
TCCACCACTG GGACAAGCCA TCTTGTAAAA TGTGCGGAGA AGGAGAAAAC TTTCTGTGTG    1020
AATGGAGGGG AGTGCTTCAT GGTGAAAGAC CTTTCAAACC CCTCGAGATA CTTGTGCAAG    1080
TAAGAAAAGA AATCCTGTGT GTCGCTTATG TCTATAACTC CTTGTTTCAG ATGATTCTAT    1140
GTCTCATGAT TGATTGTTGC TTTTTTTCCA ATTTTGTTGC ATCATGTTGA ATAATGCTGT    1200
TTTATATGTA GAGTCTTTTA AAACATTCAC ACCATTCGTC ATCACTCCTC TGTCATATGC    1260
AGTTTTGTTT TTTGCTCTTT TCAATGTGTG TGAGGTGTTT TTTGTTTTTG TTTTTGTTTT    1320
TTTGCCATGT TATTTATAGT GTTGCTTTCC TTGTGCTTTC CTTGTGGTTT TCTTGGTTGG    1380
TTATTCAGAA AAGATGTGCA GATATCACAG AGGCCTATAG CCTTTTGGTA TCTACTTCTA    1440
CATCCAATGT ATGAATTAAG CTGTAAGATA ATGTTGCTTT CTTATCCCAG TGATCACCTG    1500
CCAAATGAAT AAGACAACAA AGAGAAGCAG AAGGGCAAGA AGATTATTTA CTGACATATA    1560
TCTATTACAC TTGGGATTGT GCTTACTGTT GCATAACTAT TTTTTAAACG GAGTTTAGTT    1620
TTATATTGCT AGTAAAAAAA AAAAAAAAAA A                                   1651
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Said sequence being similar to intron 6 of the genomic heregulin sequence is a very useful tool to produce heregulin-γ specific probes for cancer diagnosis, or antisense therapy agents.

The novel heregulin variant, which is truncated in the EGF-like domain, provides a useful means to generate antibodies which do selectively recognize the specific end of the respective heregulin variant in the EGF-like domain. The heregulin needed to generate such antibodies according to known methods can be produced by recombinant methods using either the cDNA or a respective part of the genomic heregulin DNA. Antibodies can also be generated against synthetic peptides having the same sequence as HRG-γ protein sequence.

The heregulin-γ specific probes for e.g. cancer diagnostics and antisense therapy agents can be generated according to general methods known in the art such as synthetic methods.

Since in connection with breast cancer a higher concentration of heregulin-γ was found, the heregulins of the present invention provide a useful means to produce specific monoclonal antibodies. Such antibodies allow the detection of persons with an enhanced risk for breast cancer.

Another method to determine such persons is the determination of the heregulin-γ concentration by means of probes constructed on the basis of the 3' untranslated region adjacent to the exon 6 sequence, i.e. probes derived from the sequence information of nucleotides 637 to 1204 of Table I, particularly nucleotides 637 to 1186. Since nucleotides 635 and 636 are also different from the corresponding nucleotides in HRG alpha and beta, probes including these nucleotides could be used as well.

Since heregulin-γ was found to stimulate cell growth, antisense therapy agents can be prepared for e.g. breast cancer therapy, said agents being RNA or DNA antisense oligonucleotides selected from the heregulin-γ

specific region, i.e. the 3'-untranslated region, particularly from the exon 6 adjacent part of said region.

Antisense molecules can also be obtained from expression vectors (plasmids) comprising a sequence of interest introduced into the target cells where they will allow the transcription of large (up to several kilobase) RNA molecules of antisense polarity.

Since the heregulin-γ is an extracellular cell stimulator, it is a suitable tool for the detection of growth inhibitors or for the stimulation of the cell proliferation, particularly for cells lacking an ErbB receptor. Such stimulation can be performed either by recombinant techniques or by administration of the protein.

Since tamoxifen was found to stimulate the HRG-γ expression, such stimulation of the cell proliferation can be enhanced by simultaneous addition of such agents.

HRG-γ thus is also suitable as therapeutic agents for diseases connected with proliferation problems. Cells with proliferation problems are e.g. involved in osteoporosis.

Slight variations in the nucleotide sequence of the present invention still allowing the base pair formation under suitable, usually stringent, conditions are considered as falling within the scope of the present invention. Such sequences generally are about 90 % homologue to the sequence outset in Table I.

Brief Description of the Figures

Figures 1 to 3 show a partial sequence alignment of HRG-γ with HRG-α and HRG-ß and northern blot analysis of HRG-γ expression.

Figure 1 represents HRG-γ cDNA sequence (nucleotides 613 to 687 relative to the initiation codon) together with the deduced amino acid sequence (single letter code) are aligned with HRG-α and HRG-ß isoform nu-

cleic acid and peptide sequences. The stop codon in HRG-γ is marked with *. Cysteines in the EGF-like motif are shown in boldface.

Figure 2 shows the northern blot analysis of HRG-γ expression in various HBC cell lines. The northern blotting was performed using a HRG-γ specific radiolabeled probe. 20 µg total RNA were analysed. 1-2: MDA-MB-231 and MCF-7 cells (Mason Research Institute, Rockville, MD). 3-6: HS578-T, SKBRIII, ZR-75-1 and T47-D cells, respectively (American Type Culture Collection, Rockville, MD). Filled arrow: HRG-γ mRNA, open arrow : position of the 2.3kb rRNA.

Figure 3 shows detection of HRG-γ in biopsies. Total RNA was extracted from tumor tissues and subjected to RT-PCR. Upper panel: PCR amplification of GAPDH (SYBR®Green I staining). Arrow: 534 bp PCR product. Lower panel: HRG-γ detection by limited PCR and southern blot analysis. Arrow: 675 bp product. 1-10: primary tumor tissue samples obtained from 10 HBC patients.

Figures 4 to 6 show the biological activity of HRG-γ with

Figure 4 showing the ErbB-2 tyrosine phosphorylation status. SKBR-3 cells (5 x 10^7 cells) were either mock stimulated (control) or stimulated with HRG-ß1 (1 nM) or HRG-γ (30 pM) for 8 minutes at 37°C. Cells were lysed (41) and ErbB-2 was immunoprecipitated with 10 µg polyclonal ErbB-2 antibody (Santa Cruz Biotechnology, CA). Immunoprecipitates were analysed by western blotting. The ErbB-2 protein was stained by incubation of the blot with the antibody used for immunoprecipitation, whereas the ErbB-2 phosphotyrosine content was revealed with PY20 antibody (Santa Cruz Biotechnology, CA).

Figure 5 represents growth promotion by recombinant HRG-γ. MCF-7 cells were stimulated with recombinant HRG-γ and cell density was assessed after 24 hours (42). Values are expressed as OD ± sd (n = 6).

Figure 6 represents MAP kinase stimulation. MCF-7 cells (10^7 cells) were incubated in serum-free medium for 24 hours and stimulated for the time indicated either with EGF (1nM) or HRG-γ (30pM). MAP kinase activity was measured as described (43)(22) using a synthetic peptide (Amersham, UK) based on the EGF receptor Threonine^{669} phosphorylation site as substrate. MAP kinase activity is expressed as cpm ^{32}P incorporated into the substrate peptide per sample ± sd (n=3).

Figures 7 and 8 show subcellular localization of HRG-γ. MDA-MB-231 cells were electroporated with pEGFP-C1 derivatives, seeded onto sterile glass slides and analysed after 24 hours incubation without fixation.

Figure 7 depicts the HRG exons together with the restriction sites used for the construction of the deletion mutants. Hatched boxes: GFP, black box: SV40 nuclear localization sequence. Gray boxes: HRG sequences. Circles: cysteines. (a): GFP control. (b): SV40 control. (c-h): Heregulin deletion mutant sequences fused to GFP; HRG-ΔSacII, HRG-ΔSpeI, HRG-ΔBclI, HRG-ΔBbsI, HRG-ΔXmnI, HRG-ΔXhoI, respectively. (i) HRG-γ sequence fused to GFP. (j): HRG-α fused to GFP.

Figure 8 shows the fluorescence of MDA-MB-231 cells transfected with: a: pEGFP, b: pEGFP/NLS, c: pEGFP/HRG-ΔSaCII, pEGFP/HRG-ΔSpeI or pEGFP/HRG-ΔBclI, d: pEGFP/HRG-ΔBbsI, pEGFP/HRG-ΔXmnI, pEGFP/HRG-ΔXhoI, pEGFP/HRG-γ or pEGFP/HRG-α.

Figure 9 shows HRG-γ stimulation by estrogen and tamoxifen. MCF-7 cells kept on serum free medium were stimulated with 0.1nM estrogen (Fig. 9 A) or 0.1 µM Tamoxifen (Figure 9 B) for the time indicated. Total RNA (20µg) was analysed by northern blotting with a HRG-γ specific probe. Expression was determined by densitometry and is expressed as arbitrary units.

Modes for Carrying Out the Invention

cDNA Isolation and Characterization

In order to isolate heregulin-γ a cDNA library obtained from the HBC cell line MDA-MB-231 was screened and a 1.6kb clone of a novel heregulin splicing variant (see Table I) which encodes an open reading frame of 211 amino acids containing the Ig-like, glycosylation domain and part of the EGF-like domain identical with the corresponding HRG-α and ß domains (6, 17) was isolated. The library was constructed with 10 µg polyA RNA obtained from MDA-MB-231 cells. The cDNA cloning into the λZAPII vector (Stratagene, CA) was performed according to the manufacturer instructions. 10^6 clones were screened under high stringency conditions with a 336 bp probe homologous to the HRG alpha cDNA sequence (Genbank accession number M94165, nucleotides 412-747). The novel clone was isolated from the λ phages as plasmid pBluescript/HRG-γ by mass excision as described in the Statagene manual. Sequencing was either performed with the Highfidelity kit (Oncor, MD) or with the *fmol*® cycle sequencing kit (Promega, WI).

The nucleic acid sequence of said clone is represented in Table I with the coding sequence as well as the stop codon in majuscules.

In the isolated clone a stop codon interrupts the EGF-like motif after the fourth cysteine (Fig. 1). The coding sequence is flanked by a 568bp 3'-untranslated region ending with a poly(A) tail. Said heregulin variant is denominated herein furtheron HRG-γ since it is the third known sequence variation for the EGF-like motif and it is the first HRG splicing variant containing a truncated EGF-like domain.

Applying a specific probe (length 590 bp, nucleic acids 615 - 1205 isolated from plasmid pBluescript/

HRG-γ as a XhoI restriction fragment) mainly formed of the 3′ untranslated region of HRG-γ for northern blotting it was demonstrated that several HBC cell lines express a HRG-γ related 2.4kb transcript (Fig. 2). Northern blotting was performed as described (17). The probe used was a 0.6kb XhoI restriction fragment from plasmid pBluescript/HRG-γ, labeling was performed as for southern blotting. Frozen biopsies, pulverized in liquid nitrogen or cell cultures were subjected to total RNA extraction using the RNeasy kit (Qiagen, FRG). cDNA was synthesized from 1 µg total RNA at 42°C for 60 minutes with MuMLV reverse transcriptase (Promega, WI) in a 20 µl volume. The cDNA was diluted 5 fold and 1 µl was used as template for a 25 cycle control PCR reaction with primers pc1 (5′ GGTGAAGGTCGGAGTCAACGG) and pc2 (5′ GGTCATGAGTCCTTC CACGAT) that amplify a 534 bp portion of the glyceraldehyde 3 phosphate deshydrogenase (GAPDH) cDNA. The amount of GAPDH product was calculated by subjecting 5 µl control PCR to gel electrophoresis. Digital imaging of the gel stained with a 1/10,000 dilution of SYBR^{TM}Green I DNA stain (Molecular Probes, OR) was performed with the GelDoc system (BioRad, CA). The cDNA amount of each sample was calculated according to the relative amount of the GAPDH product. HRG-γ cDNA amplification was performed with primers p3 (5′ AAGTCAGAACTTCGCATTAA) and p4 (5′ CTGCACATCTTTTCTGA). PCR was carried out in buffer H (Invitrogen, CA) with 1 unit Taq DNA polymerase (Boehringer, FRG) in 50 µl reaction volume. PCR reactions were cycled 94°C 2 min, 94°C 30 sec, 55°C 30 sec, 72°C 1 min for 25 cycles with a final extension step at 72°C for 5 min. These conditions did not allow any fluorescence-based detection of amplification products (data not shown). Negative controls were made with RNA not subjected to reverse transcription and with no template. PCR products were identified by southern blotting with probes obtained by PCR with the corresponding primer pair using plasmid

pBluescript/HRG-γ as template. Labeling of probes was performed by nick translation with [α-^{32}P]-dCTP (Amersham, UK) using the Ready-to-go labeling kit (Pharmacia, Sweden). Unincorporated nucleotides were removed by column purification (Push column, Stratagene). The PCR reactions were separated by electrophoresis on 2% agarose gels, at 5 V/cm and transferred overnight by alkaline blotting to HybondN+ membranes (Amersham, UK) which were hybridized at 68°C overnight (44). The membranes were subsequently washed at 50°C for 15 min at low stringency and 42°C for 15 min at high stringency. Blots were exposed to phosphor storage screens for 1-2 days. Data were acquired with a PhosphorImager device and quantified with the ImageQuant analysis program (Molecular Dynamics, CA). The DNA sequence linked to exon 6 (6) in HRG-γ was characterized by PCR using a primer pair encompassing the stop codon to amplify genomic DNA from MDA-MB-231 and MCF-7 cells. The results revealed that the HRG-γ specific sequence corresponds to the intron located 3′ to exon 6 (herein furtheron referred to as intron 6). Primary HBC biopsies were measured for HRG-γ expression levels by semi-quantitative RT-PCR (see above) and compared to those of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control (Fig. 3). HRG-γ levels of HBC biopsies ranged from very high to low and did not correlate with the expression of HRG α/ß (data not shown), suggesting an independent transcriptional regulation of this splicing variant.

Expression of heregulin-γ in bacteria and in transfected eucaryotic cells

MCF-7 cells were transiently transfected with an expression vector carrying the HRG-γ sequence. Restriction sites were introduced in the HRG-γ sequence by PCR amplification using primers p3 (5′ TCCGTCTCCGCGAGACGGGA TCCGAGCGCAAAGAAGGC) and p4 (5′

CATAAGCGACACACAGGAT CCGTCGACGAATTCTTACAAGCACAAGTATCTC) and plasmid pBluescript/HRG-γ as template. The primer p3 introduces a BamHI site (boldface) at the site of the ATG codon whereas primer p4 introduces EcoRI, SalI and BamHI sites (boldface) 3′ to the stop codon of HRG-γ (underlined). The PCR product was cloned into plasmid pCRII (Invitrogen, CA), producing pCRII/HRG-γ. A BamHI-SalI restriction fragment was ligated into the bacterial expression vector pQE30 (Qiagen, FRG) resulting in pQE30/HRG-γ. E.coli XL1 Blue cells, transformed with plasmid pQE30/HRG-γ, were grown overnight with shaking at 37°C in LB medium supplemented with 100µg/ml ampicillin. The culture was diluted 100 fold with fresh medium and grown to an OD_{600} of 1. Recombinant protein expression was induced by addition of IPTG (0.4mM final concentration). The cells were grown for an additional 5 hours, centrifuged and the cell pellets were stored frozen at -70°C. Inclusion bodies were solubilized in 6M urea and purified over a cation exchange column (SP Sepharose Fast Flow, Pharmacia, Sweden). Pooled fractions were further batch purified in the presence of 6M urea using a silica-based nickel affinity matrix (Qiagen, FRG). Purified recombinant protein was dialyzed against 50mM Tris-HCl, 150mM NaCl, 0.1 % Tween20 and stored at 4°C.

The EcoRI-SalI region of plasmid pQE30/HRG-γ was introduced between the EcoRI and XhoI sites of the eukaryotic expression vector pCR3 (Invitrogen, CA), producing pCR3/HRG-γ. His-tagged HRG-γ was recovered in the conditioned medium (data not shown) demonstrating HRG-γ secretion in contrast to HRG-ß3 (9) lacking also the transmembrane domain.

Effects of heregulin-γ

Since the ErbB-2 receptor is activated by heterodimerization upon stimulation of any ErbB receptor (18) and since in SKBR-3 cells the ErbB-2 receptor is

overexpressed, it was tested whether HRG-γ could trigger ErbB-2 phosphorylation despite its impaired EGF-like motif. As shown in Fig. 4, recombinant HRG-γ was found to be unable to increase tyrosine phosphorylation of ErbB-2 as compared to a HRG-ß1$_{(177-246)}$ peptide (19). It was, however, found, that MCF-7 cells were growth-stimulated by recombinant HRG-γ in a dose-dependent manner (Fig. 5). Maximal response was observed at 3pM HRG-γ. In serum-deprived cells, the mitogen activated protein kinase (MAPK) p42/p44 involved in most growth factors (20) and HRG signaling pathways (21,22) was significantly stimulated by 30pM HRG-γ (Fig. 6) demonstrating that cell stimulation by recombinant HRG-γ occurs with an ErbB-2 independent signaling pathway suggesting an intracellular function.

In this context, it is known that exogenously applied HRG-ß1 is rapidly imported into the nucleus of SKBR-3 cells (23). In addition, nuclear localization of several polypeptide growth factors was demonstrated in target cells (24,25,26,27) and the requirement for nuclear localization sequences (NLS) to achieve mitogenic activity was reported (27,28). Furthermore basic fibroblast growth factor (bFGF) was described to be able to regulate transcription in a cell free system (29). It was thus desired to get further information on the behaviour of HRG-γ by analyzing its subcellular localization and mapping the internal HRG-γ amino acid sequence responsible for compartmentalization. A HRG-γ-green fluorescent protein (GFP) (30) expression vector was used to transfect MCF-7 cells. An enhanced Green Fluorescent Protein (EGFP) fusion expression vector was obtained by inserting the BamHI-SalI restriction fragment from pQE30/HRG-γ into the BglII and SalI sites of plasmid pEGFP-C1 (Clontech, CA), in frame with the EGFP coding sequence, resulting in pEGFP/HRG-γ. HBC cell lines were transfected by electroporation using the Gene Pulser II (BioRad). 10^7 cells were trypsinyzed, resuspended in 500 µl complete growth medium with 20-40 µg plasmid DNA and subjected to a sin-

gle pulse (1350) V/cm, 960 µF, no parallel resistance). The cells were kept on ice for 10 min, resuspended in complete growth medium and seeded on sterile glass slides. After 24 hour, unfixed cells were observed with a Zeiss Axioskop microscope. GFP fluorescence was observed with FITC filters. Image acquisition was performed with a CCD camera using the MacProbe program (Perceptive Scientific Instruments Inc., UK). Control experiments with GFP showed after 24 hours a distribution of this marker throughout the whole cell body, including the nucleus and a distinctly fainter staining of the nucleoli (Fig. 8, a). The fusion of the SV40 large T antigen (T-ag) NLS to GFP induced nuclear import of the fusion protein (Fig. 8, b) with little efficiency since the minimal T-ag NLS sequence used (PKKKRKV) mediates import with a slow kinetic (31). Fusion of the HRG-γ coding sequence to the GFP resulted in the exclusive nuclear localization of this protein. Nucleolar staining was observed together with a weaker nucleus staining (Fig. 8, d), or a dot-like localization throughout the whole nucleus. The fluorescence intensity of the dots appeared inversely correlated to the corresponding nucleolus fluorescence. The same nuclear localization was also detected for a fusion construct encoding the extracellular part of HRG-α (Fig. 8, d). The results are consistent with the observation that heregulins have the capacity to enter nuclei (23).

The amino acid domains responsible for nuclear import were mapped by sequential 3′ deletions of the HRG-γ sequence in the GFP fusion constructs (see Fig. 7) in the following manner. GFP-fusion deletion mutants were obtained as follows: pEGFP/HRG-ΔBbsI and pEGFP/HRG-ΔSpeI were constructed from plasmid pEGFP/HRG-γ by linearisation at the appropriate restriction sites, fill-in with T4 DNA polymerase and subsequent digestion with SmaI and religation. Deletion mutant pEGFP/HRG-ΔXmnI was obtained by double digestion with XmnI and SmaI and religation. Deletion mutants pEGFP/HRG-ΔXhoI, pEGFP/HRG-ΔBclI

were obtained by double digestion with XhoI/SalI and BamHI/BclI respectively. The pEGFP/HRGΔSacII construct was obtained by SacII digestion and subsequent religation. pEGFP/HRG-α was obtained by prior isolation of a HRG-α PCR product obtained from MDA-MB-231 cell cDNA : With the primer pair p7 (3'AAACTAGTCCTTCGGTG) and p8 (3'GGAATTCACATGA TGCCGACCACAAGGA) a 632 bp fragment was amplified, cloned into pCRII. The resulting pCRII/HRG-α plasmid was cut with EarI, end-blunted and digested with SpeI. A 564bp fragment was isolated and cloned into a SpeI/SmaI digested pEGFP/HRG-γ plasmid, resulting in pEGFP/HRG-α. Plasmid pEGFP/NLS was obtained by ligating a phosphorylated synthetic oligonucleotide linker (pN1 3'TCGATATCCAAAGAAGA AGCGCAAGGTGCA and pN2 3' CCTTGCGCTTCTTCTTTGGATA) into plasmid pEGFP-C1 digested with XhoI and PstI. The transfection of these plasmids revealed that the N-terminal 25 amino acid region of heregulins (encoded up to the SacII site) are importing the GFP into the nucleus/nucleolus similar to the SV40 control (Fig. 8, panel b and c). This domain contains stretches of positively charged amino acids potentially serving as NLS (4). A striking change in the nuclear import pattern was obtained when the BclI-BbsI region was present in the fusion construct (Fig. 8, compare c with d) yielding a nuclear localization as observed with the HRG-γ or HRG-α GFP fusion constructs, demonstrating that the BclI-BbsI region either enhanced nuclear import via the N-terminal NLS or mediated itself nuclear import. Alternatively, the BclI-BbsI domain may exert a specific biological function within the nucleus (Fig. 8, d). The subnuclear localization obtained with HRG sequences fused to GFP (Fig. 8, d) is reminiscent of the cell cycle dependent, nuclear dot pattern observed with human Rad51 (32) and BRCA1 distribution (33) and are consistent with the findings that HRG alter the cell cycle (15, 34, 35).

The intracellular localization of HRG and the lack of ErbB-2 activation by HRG-γ show that cancer

therapies aimed at blocking the ErbB-2 receptor are not sufficient since such splicing variants bypass this mechanism. In addition, it is known that ErbB-2 receptor activation down regulates the estrogen receptor (ER) (36, 37). HRG expression could also be involved in the endocrine treatment failure of HBC by down modulating the ER expression as well. Reciprocal expression of HRG by activation of steroid receptors might be of equal importance. Therefore the effect of estrogen and the antiestrogen tamoxifen on the expression levels of HRG-γ was investigated. Northern blot analysis revealed that MCF-7 cells respond to both estradiol (0.1 nM) and tamoxifen (0.1µM, corresponding to serum concentration of patients under standard Tamoxifen therapy) with a significant increase in HRG-γ transcript, reaching a maximum level 12h after stimulation (Fig. 9). These results give strong evidence that non-steroidal antiestrogens, such as tamoxifen, are able to stimulate the expression of HRG-γ in a set of HBC cells thereby neutralizing the antiestrogenic effect by stimulating a mitogenic activity which could implicate either antiestrogen resistance or carcinogenic side effects (38).

In conclusion, these findings reveal that a molecule with a truncated EGF-like motif such as HRG-γ triggers a biological response on cells, such as e.g. target MCF-7 cells, without activating ErbB-2. Tyrosine kinase-independent signaling was also reported for SDGF (Schwannoma-derived growth factor), able to act as a mitogen even with a dysfunctional EGF receptor (28). Furthermore, tyrosine kinase-defective receptors promoted MAPK activation (18, 39) and c-myc induction (40). A similar mechanism may be responsible for the HRG-γ signaling, circumventing ErbB-2 activation.

Diagnostic and therapeutic applications

Due to its biological effects, heregulin-γ is a suitable agent for the treatment of diseases connected with reduced cell proliferation such as e.g. osteoporosis. Such treatment can be performed by direct administration of the heregulin or by transfection of heregulin deficient cells.

Heregulin-γ is also a very useful means to produce selective antibodies due to the truncated EGF-domain resulting in a heregulin-γ-specific recognition site. Such antibodies themselves are very useful diagnostic agents, allowing the determination of e.g. persons with an enhanced risk for breast cancer.

Another agent for the determination of e.g. persons with an enhanced risk for breast cancer is an oligonucleotide comprising a sequence, which is at least partially derived from the 3'-untranslated region adjacent to exon 6 of the heregulin γ-sequence of Table I. Said sequence of the oligonucleotide, which is derived from the 3'untranslated region addressed above hybridizes under stringent conditions with the heregulin-γ RNA. Such sequences generally are about at least 90 % homologue to the respective sequence of Table I.

Heregulin-γ can also be used to evaluate and search for growth inhibitors suitable as effective substances in cancer therapy, e.g. alone or in combination with other effective substances.

Site directed mutagenesis of the coding sequence allows to produce recombinant proteins that use the same pathway (bind to the same receptor(s)) but do not trigger downstream signalling, thereby competing with HRG-γ.

The cDNA sequence of Table I provides an important means for the production of selective antisense therapy agents which - for good selectivity - are made on the basis of the 3'-untranslated region, preferably of the intron 6 like sequence adjacent to exon 6.

Non-therapeutic uses

Heregulin-γ can also be used to generally stimulate cells lacking an ErbB receptor either as therapeutical agent or in cell cultures. Besides of the administration of the protein itself, cells can be transfected with the heregulin-γ DNA.

Additionally, it was found that the Ig-like domain is a very important sequence for the nuclear or subnuclear localization. This sequence thus can be used as such or modified to regulate cell active processes. Such processes can e.g. be the heregulin-γ production, which can be regulated by modifications performed in the Ig-like domain such as deletions, substitutions etc., or the Ig-like domain or its nucleotide sequence, respectively, can as such or in modified form be connected with suitable cell active proteins or nucleotide sequences thereof, respectively.

Independent of their use, the oligonucleotides of the present invention usually have an interesting part of a length of 10 or more bases. In this range a homology about 90 % usually is sufficient to get hybridization under suitably selective conditions. "Interesting part" means a contiguous part of the oligonucleotide that is intended to hybridize with the sequence of interest. It is general knowledge that hybridization is obtained if at least one contiguous part(s) of the oligonucleotide has at least about 90 % homology with a sequence of interest and that the selectivity is enhanced with longer "interesting parts".

Oligonucleotides can be made by chemical or enzymatic synthesis. Large nucleotide sequences advantageously can be made by current Polymerase Chain Reaction techniques.

An Ig-like domain or modified Ig-like domain comprising protein can also be used for the nuclear import of cell active molecules.

While there are shown and described presently preferred embodiments of the invention, it is to be distinctly understood that the invention is not limited thereto but may be otherwise variously embodied and practiced within the scope of the following claims.

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Claims

1. A nucleotide sequence encoding amino acids 1 to 211 of heregulin comprising

a) the coding DNA sequence nucleotides 1 to 636, set out in Table I,

b) the RNA sequence corresponding to a) or

c) a DNA or RNA sequence which, but for the degeneracy of the genetic code, would hybridize to (is identical with) the coding sequence of a) or b) under (most) stringent conditions.

2. The nucleotide sequence of claim 1 wherein the 3′ end of the nucleotide sequence defined in a), b) and c) is linked to an untranslated nucleotide sequence which 3′-untranslated sequence represents at its 5′-end at least part of the nucleotide sequence corresponding to intron 3′ of exon 6 of heregulin or a sequence which under stringent condition hybridizes to the respective nucleotide sequence of intron 3′ to exon 6 of heregulin or a part thereof (or a sequence which represents at its 5′-end at least part of a sequence which is to at least 90 % homologue to the nucleotide sequence corresponding to intron 3′ of exon 6 of heregulin).

3. The nucleotide sequence of one of claims 1 or 2 comprising a 5′-untranslated region which as such would hybridize to the DNA sequence set out in Table I or the respective RNA sequence.

4. A nucleotide sequence of anyone of claims 1 to 3 which comprises the cDNA sequence set out in Table I or the respective RNA sequence.

5. A genomic DNA sequence encoding an amino acid sequence consisting of amino acids 1 to 211 of heregulin-α and heregulin-ß.

6. The nucleotide sequence which is the complementary strand of any of the nucleotide sequences defined in one of claims 1 to 5.

7. A heregulin consisting of amino acids 1 to 211 of heregulin-α and heregulin-ß.

8. The heregulin of claim 7 which is a recombinant heregulin.

9. A process for the production of the recombinant heregulin of claim 8 comprising the expression of a DNA sequence of any one of claims 1 to 6 in a eucaryotic or procaryotic host cell.

10. A oligonucleotide sequence comprising a partial sequence corresponding to at least a part of the 3'-untranslated region outset in Table I or being at least 90 % homologue to the respective region of Table I or the respective RNA sequences.

11. The oligonucleotide sequence of claim 10 at least a part of which is selected within nucleic acids 637 - 1207 of Table I.

12. A diagnostic or therapeutical composition comprising an oligonucleotide of anyone of claims 10 or 11.

13. A process for the production of oligonucleotide sequences according to anyone of claims 10 or 11 wherein the nucleotide sequence is obtained by chemical or enzymatic synthesis or by Polymerase Chain Reaction techniques.

14. An antibody for the heregulin consisting of amino acids 1 to 211 being selective to the C-terminal amino acids.

15. A pharmaceutical composition comprising as effective substance a heregulin, the amino acid sequence of which consists in amino acids 1 to 211 of heregulin-α and heregulin-ß, particularly a pharmaceutical composition for the treatment of diseases caused by reduced cell proliferation.

16. Use of a heregulin the amino acid sequence of which consists in amino acids 1 to 211 of heregulin-α and heregulin-ß for the in vitro stimulation of cells.

17. A heregulin consisting of a modified amino acid sequence 1 to 211 for the regulation of processes connected with natural heregulin.

18. Use of a Ig-like domain or a modified Ig-like domain comprising protein for the regulation of cell active processes.

19. A process for the production of a nuclear or subnuclear localizable cell active protein, characterized in that said protein is connected with an Ig-like domain or a modified Ig-like domain by chemical synthesis or by expression of a respectively connected DNA sequence.

20. Use of a Ig-like domain or a modified Ig-like domain comprising protein for the nuclear import of cell active molecules.

HRG-α

613 CCCTCGAGATACTTGTGCAAGTGCCAACCTGGATTCACTGGAGCAAGATGTACTGAGAATGTGCCCATGAAAGTCCAAAAC...
205 P S R Y L C K C Q P G F T G A R C T E N V P M K V Q N ...

HRG-β

613 CCCTCGAGATACTTGTGCAAGTGCCCAAATGAGTTTACTGGTGATCGCTGCCAAAACTACGTAATGGCCAGCTTCTACAAG...
205 P S R Y L C K C P N E F T G D R C Q N Y V M A S F Y K .

HRG-γ

613 CCCTCGAGATACTTGTGCAAGTAAgaaaagaaatcctgtgtcgcttatgtctataactccttgtttcagatgattctat...
205 P S R Y L C K *

Fig. 1

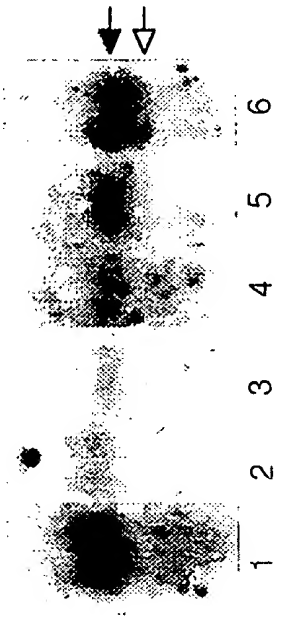


Fig. 2

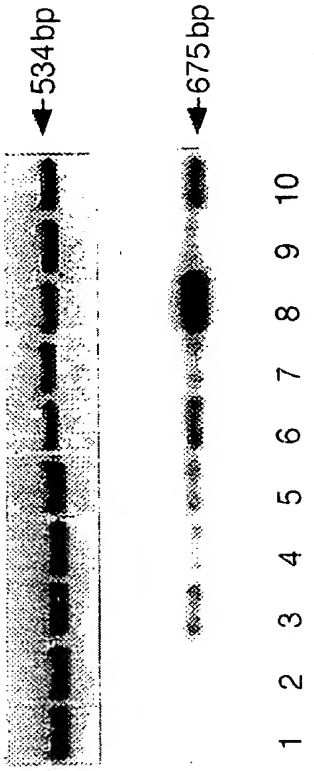


Fig. 3

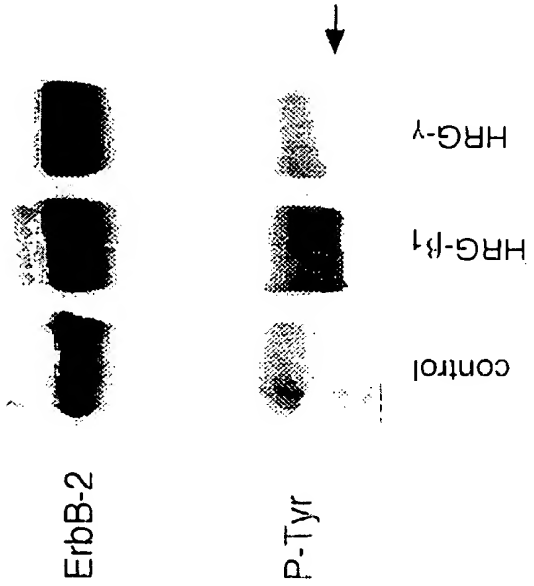


Fig. 4

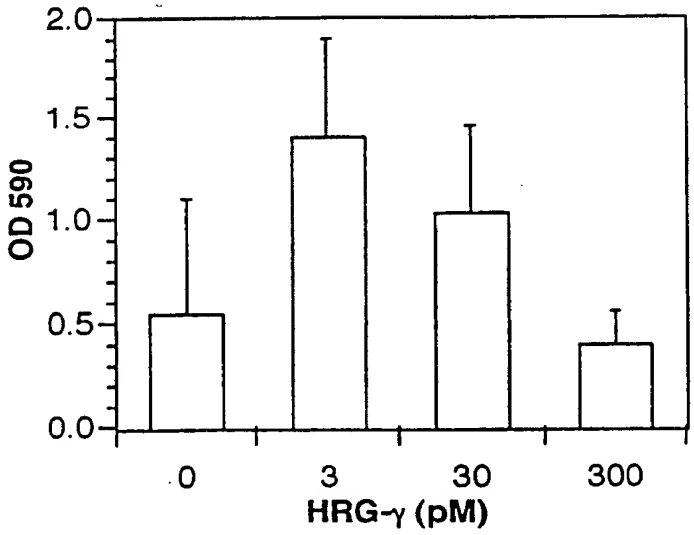
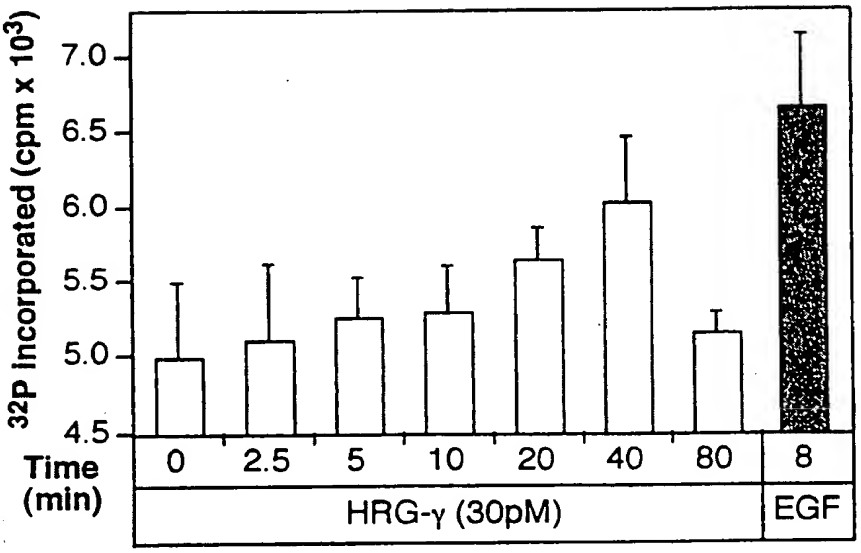


Fig. 5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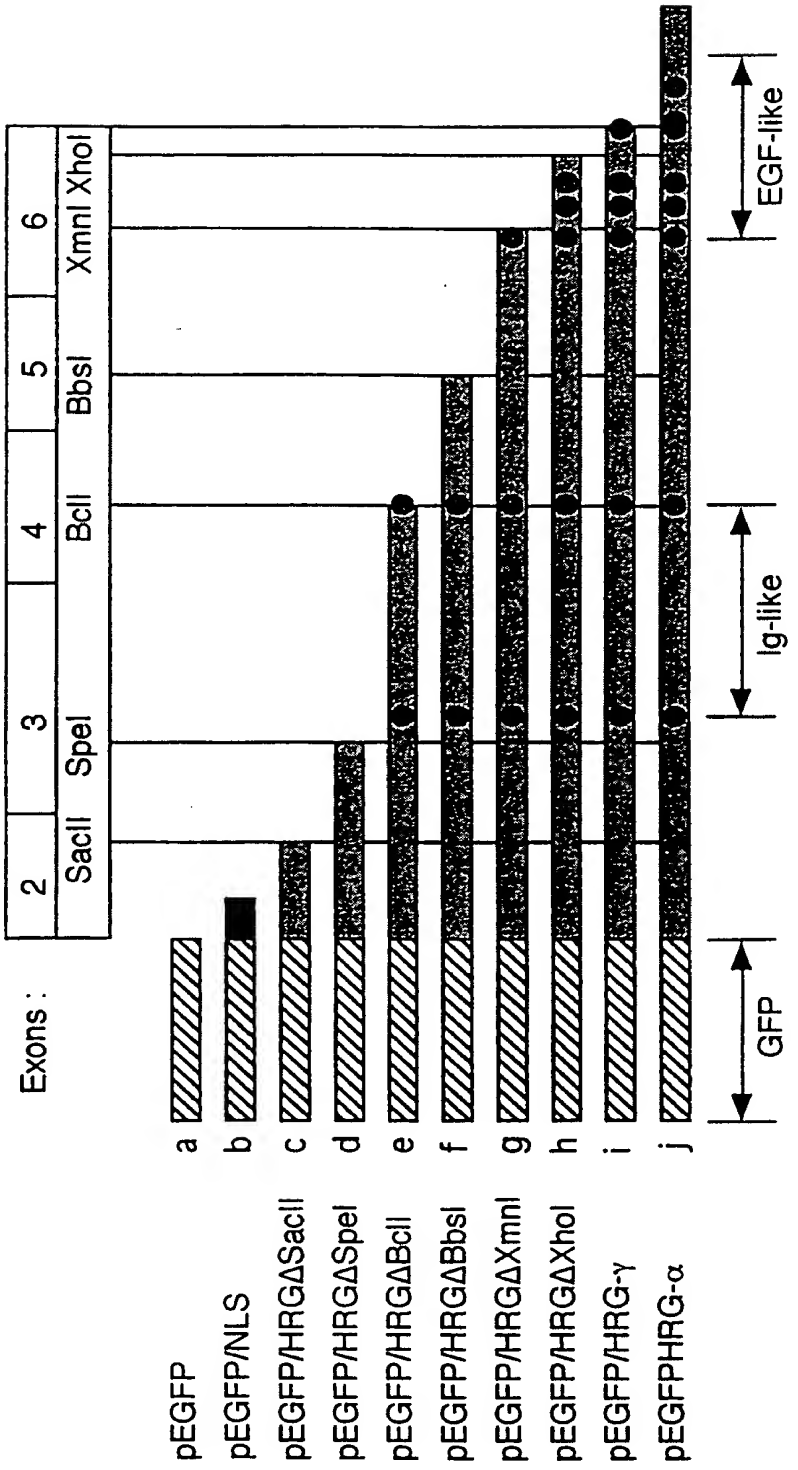
6.5
6.0
5.5
5.0
4.5
Time (min)
0
2.5
5
10
20
40
80
8
HRG-γ (30pM)
EGF

Fig. 6


Exons :
2
3
4
5
6
SacII
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XmnI XhoI
a
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g
h
i
j
pEGFP
pEGFP/NLS
pEGFP/HRGΔSacII
pEGFP/HRGΔSpeI
pEGFP/HRGΔBclI
pEGFP/HRGΔBbsI
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Fig. 7

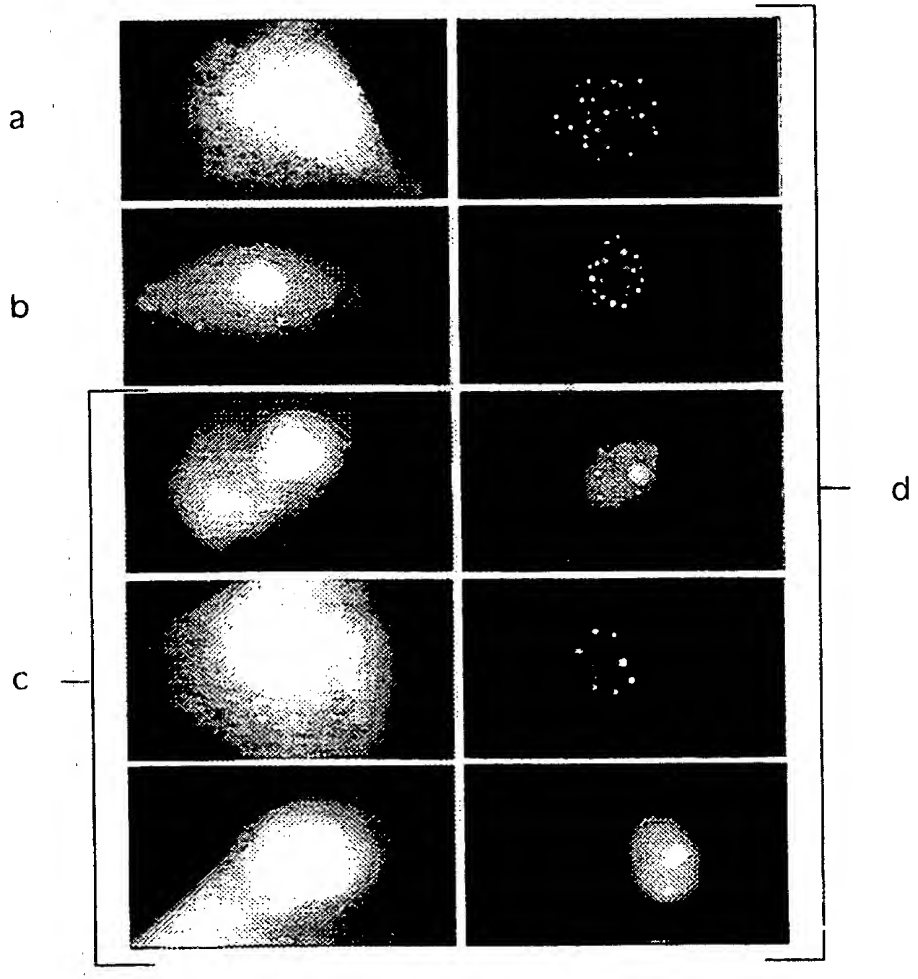

Fig. 8

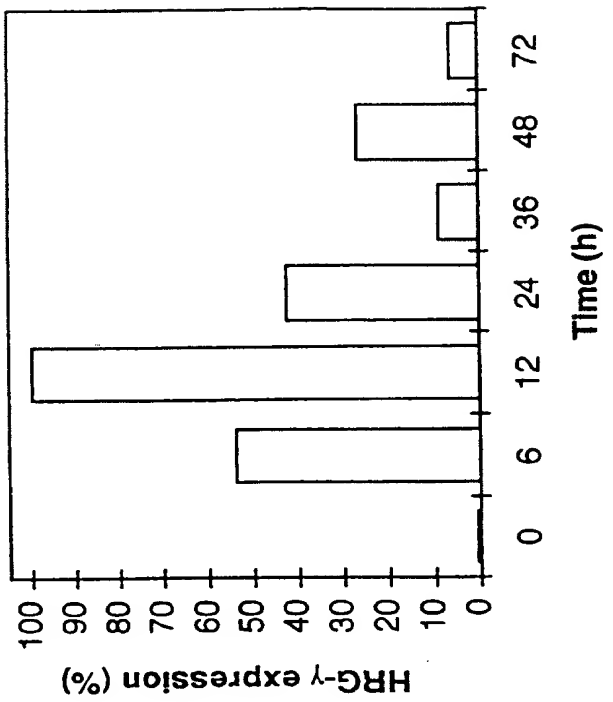
HRG-γ expression (%)
100
90
80
70
60
50
40
30
20
10
0
0
6
12
24
36
48
72
Time (h)

Fig. 9 B

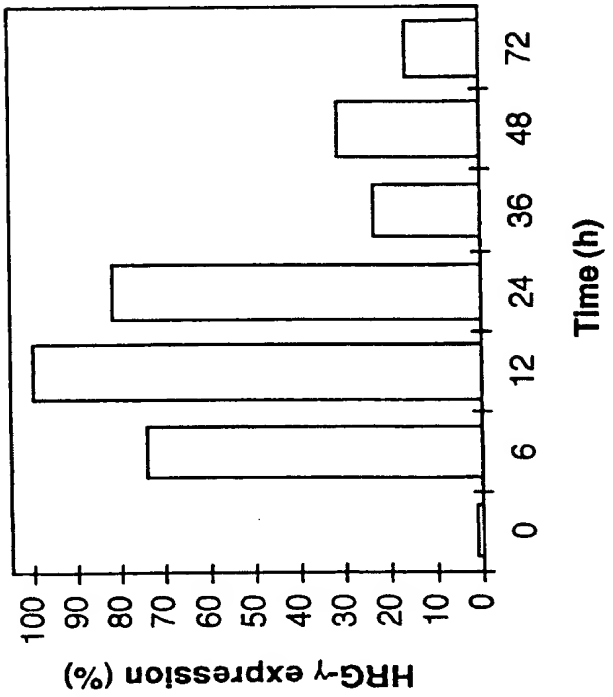
HRG-γ expression (%)
100
90
80
70
60
50
40
30
20
10
0
0
6
12
24
36
48
72
Time (h)

Fig. 9 A

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/IB 97/01287

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/475 C12Q1/68 A61K48/00 C07K16/22
A61K38/18 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCHAEFER, GABRIELE ET AL: ". gamma.-Heregulin: a novel heregulin isoform that is an autocrine growth factor for the human breast cancer cell line, MDA-MB-175" ONCOGENE (1997), 15(12), 1385-1394 CODEN: ONCNES;ISSN: 0950-9232, 1997, XP002064745 see abstract see page 1386, left-hand column, paragraph 2 - page 1387, left-hand column, paragraph 3 see page 1388, left-hand column, paragraph 2 - page 1392, left-hand column, paragraph 2 ---	1-17
A	WO 94 28133 A (AMGEN INC.) 8 December 1994 see examples 7,13 --- -/--	1-17

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
13 May 1998	18. 08. 1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	MONTERO LOPEZ B.

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/IB 97/01287

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DUANZHI WEN ET AL.: "Structural and functional aspects of the multiplicity of Neu differentiation factors" MOLECULAR AND CELLULAR BIOLOGY, vol. 14, no. 3, March 1994, WASHINGTON US, pages 1909-1919, XP002064746 cited in the application see abstract see page 1912, right-hand column, paragraph 3 - page 1917, left-hand column, paragraph 2 see page 1918, left-hand column, paragraph 2 ---	1-17
A	DUANZHI WEN ET AL.: "Neu differentition factor: A transmembrane glycoprotein containing an EGF domain and an Immunoglobulin homology unit" CELL, vol. 69, 1 May 1992, NA US, pages 559-572, XP002002185 cited in the application see abstract see page 560, left-hand column, paragraph 3 - page 568, right-hand column, paragraph 2 ---	1-17
A	WO 95 02052 A (F. HOFFMANN-LA ROCHE AG) 19 January 1995 see the whole document -----	1-17

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

national application No.
PCT/IB 97/01287

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see continuation-sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-17

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-17

   Nucleotide sequence encoding amino acids 1 to 211 of heregulin, oligonucleotide and amino acid sequence thereof, process for production thereof, corresponding antibody; diagnostic composition comprising the oligonucleotide and pharmaceutical composition containing the heregulin, and use thereof for in vitro stimulation of cells.

2. Claims: 18-20

   Use of a Ig-like domain comprising protein for regulation of cell active processes and nuclear import of cell active molecules.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/IB 97/01287

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9428133 A	08-12-94	AU 7042994 A	20-12-94
WO 9502052 A	19-01-95	AU 7185894 A	06-02-95
		CA 2141417 A	19-01-95
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		EP 0659212 A	28-06-95
		JP 8501456 T	20-02-96
		NZ 268592 A	25-09-96
		ZA 9404687 A	24-02-95